mTOR complex 2 signaling and functions

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The mechanistic target of rapamycin (mTOR) plays a central role in cellular growth and metabolism. mTOR forms two distinct protein complexes, mTORC1 and mTORC2. Much is known about the regulation and functions of mTORC1 due to availability of a natural compound, rapamycin, that inhibits this complex. Studies that define mTORC2 cellular functions and signaling have lagged behind. The development of pharmacological inhibitors that block mTOR kinase activity, and thereby inhibit both mTOR complexes, along with availability of mice with genetic knockouts in mTOR complex components have now provided new insights on mTORC2 function and regulation. Since prolonged effects of rapamycin can also disrupt mTORC2, it is worth re-evaluating the contribution of this less-studied mTOR complex in cancer, metabolic disorders and aging. In this review, we focus on recent developments on mammalian mTORC2 signaling mechanisms and its cellular and tissue-specific functions.

Introduction

The mechanistic target of rapamycin (mTOR), as the name implies, is a protein kinase that is a specific target of the natural compound rapamycin. Early studies in yeast and mammals have shown that TOR functions become inhibited by binding of TOR to rapamycin that is in complex with the prolyl isomerase FKBP12.[1-3] However, not all TOR functions are inhibited by this compound.[4,5] The identification of distinct mTOR protein complexes, mTORC1 and mTORC2, has shed light on these earlier puzzling observations and revealed the many cellular functions of mTOR. mTOR complex 1 (mTORC1), consisting of the conserved components mTOR, raptor and mLST8, binds and is inhibited by rapamycin.[6-8] It responds to the presence of nutrients and energy sources to control protein synthesis. Why not all mTOR functions can be inhibited by rapamycin is not fully understood, but some of these functions could be mediated by mTORC2. Several recent reviews have covered the regulation, signaling and rapamycin-sensitive and -insensitive functions of mTORC1.[9-12] The availability of pharmacological inhibitors that inhibit both mTORC1 and mTORC2 along with the generation of animal models that disrupt mTORC2 in specific tissues have allowed more detailed characterization of mTORC2. Because

mTORC2 is a critical regulator of Akt, a protein that is often deregulated in cancer, this mTOR complex is gaining attention as a relevant target for cancer therapy.[13,14] In this review, we will discuss the recent advancements on the regulation and functions of mTORC2.

mTORC2 Assembly and Component Phosphorylation

The core components of mTORC2 that are conserved from yeast to man include mTOR, rictor (mAVO3), SIN1 and mLST8 (GβL). Other less-conserved proteins have been found to associate with mTORC2, such as PRR5/Protor, PRR5L[15-17] and DEPTOR[18] (**Fig. 1**). mTOR is a member of a family of protein kinases termed the PIKKs (phosphatidylinositol-3-kinase-related kinases), which function as serine/threonine protein kinases. Rictor lacks known common motifs but contains a C terminus that is conserved among vertebrates. SIN1 undergoes alternative splicing and generates five isoforms.[19,20] The two longest isoforms contain a pleckstrin homology (PH) domain that has been shown to bind lipids and a Ras-binding domain (RBD) that can bind activated Ras.[21] At least three distinct mTORC2 complexes can be formed independently by three of these isoforms,[20] but unique functions for each of the isoforms have not been reported. mLST8 is made up of WD40 repeats and can bind to mTOR near the kinase domain.

Protor/PRR5 interacts with rictor even under mTORC2-disrupted conditions. The expression of PRR5 is also regulated by rictor, and in some cancers, such as colorectal and breast carcinomas, its mRNA levels become elevated.[22] DEPTOR contains two DEP (disheveled, egl-10, pleckstrin) domains and a PDZ (postsynaptic density 95, discs large, zonula occludens-1) domain.[18] It interacts with the C-terminal portion of mTOR that is upstream of its kinase domain and can bind to either mTORC1 or mTORC2. DEPTOR expression is regulated by mTORC1 and mTORC2 at the transcriptional and post-translational level. Loss of DEPTOR activates mTORC kinase activities, indicating that it negatively regulates both mTORCs. However, DEPTOR is found to be highly overexpressed in a subset of multiple myelomas, and this overexpression maintains PI3K and Akt activation, suggesting that, in these cancers, DEPTOR can activate mTORC2 signaling.

The stability and integrity of mTORC2 is dependent on the presence of the core subunits rictor and SIN1. Deficiency in rictor expression results in reduced SIN1 levels and vice versa, whereas the level of mTOR remains unchanged.[20,23-25] The tight
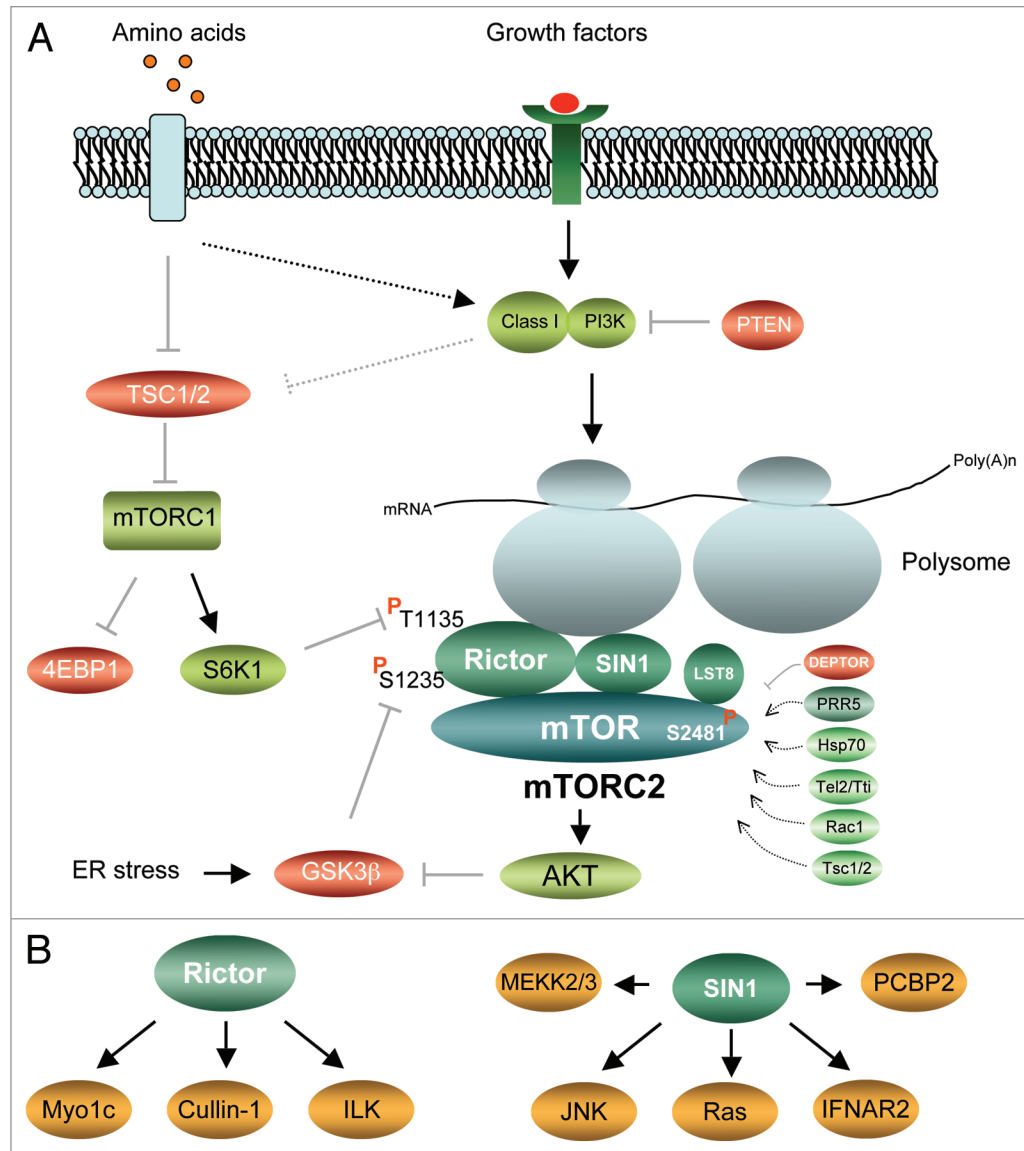
Figure 1. mTORC2 components, interactors and upstream regulators (A) mTORC2, consisting of the core components, mTOR, rictor, SIN1, mLST8, responds to the presence of growth signals. It associates with the large subunit of ribosomes. mTORC2 also associates with other less-conserved proteins that could regulate specific functions of mTORC2. (B) Rictor and SIN1 associates with other proteins independently of mTORC2. Positive regulators are shaded in green, while negative regulators are in red. TSC1/2, a negative regulator of mTORC1, was shown to be required for mTORC2 activity, hence is also shown in green.

interaction of rictor and SIN1 suggests they require each other for stability.[25,26] Reduction of rictor or SIN1 expression also disrupts mTORC2 complex assembly. Deficiency in mLST8 impairs the association of rictor with mTOR but does not affect raptor and mTOR[24] nor rictor and SIN1 association.[27] Thus, mLST8 may not be required for protein stability of rictor and SIN1. Even though mLST8 is present in both mTORC1 and mTORC2, it is required for mTORC2 functions but not mTORC1 signaling to S6K and 4E-BP.[24] Rictor and SIN1 that are found associated with mTOR are hyperphosphorylated, suggesting that phosphorylation may modulate their interaction with mTOR, or mTOR itself could phosphorylate its partners.[25,28]

TOR can form dimers or multimers. Cryo-electron microscopy confirmed that mTORC1 forms a dimer.[29] The dimerization state is sensitive to nutrients but insensitive to growth factors.[30,31] In yeast, TORC2 is oligomeric and likely forms TORC2-TORC2 dimers,[32] but there are conflicting reports as to whether mTORC2 can form dimers/multimers.[20,31] Dimerization may facilitate inter- and intramolecular phosphorylation of other complex components or substrates.

Other factors have been reported to affect mTORC2 assembly. Hsp70 interacts with rictor, and its knockdown reduces rictor levels as well as mTOR-rictor interaction, resulting in impaired mTORC2 formation and activity.[33] The maturation and assembly

of mTORCs was also shown to be dependent on Tel2 and Tti.[34,35] Tel2 and Hsp90 mediate the formation of both TORCs along with assembly of other PIKKs.[36,37] Whether mTORC2 signaling can be modulated via Tel2-mediated assembly of mTORC2 remains to be examined. Chronic treatment with rapamycin, which binds to mTOR as part of mTORC1, can prevent de novo assembly of mTORC2 and, consequently, reduces mTORC2 levels in many cell lines.[38] Under this condition, rictor and SIN1 levels are not perturbed, perhaps due to undisrupted interaction between rictor and SIN1. The sensitivity of mTORC2 to extended rapamycin treatment can be augmented by suppressing phospholipase D (PLD) activity.[39] The PLD metabolite phosphatidic acid (PA) interacts with the FKBP12/rapamycin binding domain of mTOR. Rapamycin-FKBP12 complex is thought to compete against PA for binding to this domain.[40] Thus, lower concentrations of rapamycin are required to disrupt mTOR complexes when PA levels are diminished. mTOR inhibitors that target the active site, such as Torin1, have no effect on the stability of either mTORC1 or mTORC2.[41]

mTOR can be directly regulated via phosphorylation. Some of these phosphosites are induced by growth signals. mTOR becomes autophosphorylated at Ser2481, and this phosphorylation is insensitive to amino acid or serum withdrawal but is abolished upon wortmannin treatment[42] or prolonged rapamycin treatment.[43] More recently, phosphorylation of this site was demonstrated to be a marker for intact mTORC2.[43] Some cancer cell lines wherein Akt Ser473 phosphorylation was increased or unchanged upon prolonged rapamycin treatment displayed a reduction in Ser2481 phosphorylation, correlating with disruption of mTORC2 assembly. Thus this phosphosite is a better marker for mTORC2 activity than Akt Ser473 phosphorylation. In another study, mTOR phosphorylated at Ser2481 was also found in raptor immunoprecipitates and was sensitive to acute rapamycin treatment, in contrast to rictor immunoprecipitates.[44] Phospho-Ser2481, along with the pSer2448, a site that is predominantly phosphorylated in mTORC1, conserved among vertebrate species but absent in invertebrates.[43] Since the insulin-induced phosphorylation of Ser2481 is blocked by PI3K inhibitors, and mTORC2 in vitro kinase activity is also reduced by this treatment,[45] regulation at this site may have evolved in vertebrates in response to growth factors. Ser1261 is also phosphorylated in both mTORCs in an insulin/PI3K-dependent manner.[46] It is not known if phosphorylation of this site can modulate a specific mTORC2 function. Other mTOR phosphosites, such as Ser2454, Thr2473/Thr2474, were identified by mass spectrometry, but the function remains unknown.[47]

Rictor is phosphorylated at multiple sites. By mass spectrometry analyses and comparison with phospho-proteome data sets, up to 37 phosphosites could be identified in rictor.[48] The vast majority of these sites were found in the C-terminal half of rictor.[48-50] Since the sequence in this region is conserved among vertebrates but poorly conserved in lower eukaryotes, it suggests that regulatory mechanisms evolved later that could impart a more diverse function for rictor. One of these sites, Thr1135, lies within a motif predicted to be phosphorylated by AGC kinases (Protein kinase A, PKG, PKC). This site is phosphorylated by

S6K1 and binds 14-3-3 proteins when phosphorylated.[48-50] The function of this phosphorylation is uncertain, but if mutated to Ala, expression of this mutant can modestly increase Akt phosphorylation at Ser473.[48,49] Other studies failed to detect changes in growth factor-dependent phosphorylation of Akt.[50,51] Furthermore, total expression of mTORC2 targets SGK1 and PKCα, along with phosphorylation of the SGK1 target NDRG1 were not affected by mutation of this phosphosite. Thus, it was proposed that phosphorylation of this site by S6K1 could serve as a feedback control to mTORC2 by mTORC1. Since Thr1135 is an amino acid-, growth factor-regulated site and is acutely sensitive to rapamycin, this further supports that mTORC2 is regulated by these growth signals. However, this phosphorylation can be observed in SIN1-null MEFs, suggesting that it could play a role in mTORC2-independent function of rictor.[51] Indeed, phosphorylation of Thr1135 was also shown to disrupt the interaction of rictor with cullin-1 and to reduce the ability of the rictor/cullin complex to ubiquitinate SGK1.[52] Rictor has been found to associate with other proteins independently of mTOR, such as cullin-1, Myo1c and integrin-linked kinase (ILK), supporting the notion that it could mediate other functions outside of mTORC2.[52-54] Rictor is also phosphorylated at Ser1235 by GSK3β during endoplasmic reticulum (ER) stress.[55] This phosphorylation interfered with Akt-mTORC2 binding and mTORC2 downstream signaling. These findings further substantiate that mTORC2 activity could be negatively regulated via rictor phosphorylation.

SIN1 is also phosphorylated, most likely at multiple sites, but these sites remain to be identified. Hypophosphorylated SIN1 has low affinity for mTOR[25] but can interact with rictor.[56] Prolonged rapamycin treatment induces complete dephosphorylation of SIN1, leading to cytoplasmic translocation of nuclear rictor and SIN1.[56] SIN1 has also been reported to associate with other proteins independently of mTOR and rictor. Remarkably, these proteins are involved in stress responses, including JNK, MEKK2, ATF2, p38, ras and the stress-related cytokine receptors IFNAR2, TNFR1 and TNFR2.[19,21,57-59] DEPTOR contains several phosphorylation sites that are followed by Pro residue(s), but the function of these phosphosites remains to be determined.[18]

Upstream Regulators

Whereas much is known about how mTORC1 can be regulated by upstream signals, such as amino acids and growth factors, we are only beginning to understand how mTORC2 activity can be triggered by these signals (**Fig. 1**). The mTORC2-mediated phosphorylation of Akt at the hydrophobic motif (HM) site (Ser473) denoted that mTORC2 is activated by growth factors. Indeed, in vitro kinase assays have provided evidence to support this notion.[20,25,60] Lately, the addition of phosphatidylinositol-3,4,5-trisphosphate (PIP3) to mTORC2 kinase assays was shown to enhance the phosphorylation of Akt in vitro. This could be due to a conformational change in Akt by binding of the PH domain with PIP3, which facilitates efficient phosphorylation. Additionally, it was proposed that mTORC2 may be directly activated by this phospholipid.[61] Because mTORC2 can also mediate the phosphorylation of Akt at the turn motif (TM; Thr450) site,

which is not inducible by growth factors, the divergent regulation of these two sites by mTORC2 presented a puzzle.[62,63] However, recent findings provide new insights that can resolve this issue. The phosphorylation of the TM site occurs during the translation of nascent Akt. mTORC2 mediates this phosphorylation by associating with translating ribosomes.[64] Hence, this would imply that mTORC2 activity towards Akt TM phosphorylation is not constitutive, but is regulated by signals that promote Akt translation or mTORC2 association with ribosomes. That TM phosphorylation is regulated would also be consistent with the finding that mTORC2 and ribosome interaction is stimulated by insulin-PI3K signals and that this interaction promotes mTORC2 activation.[65] In cancer cells that have elevated PI3K signaling, the mTORC2-ribosome binding is enhanced. Precisely how mTORC2 can be activated by its association with ribosomes remains to be elucidated. Because an intact ribosome seems to be required for mTORC2 activity, the ribosome could serve as a scaffold to allow mTORC2 phosphorylation of its substrate or could serve to localize mTORC2 to the proper compartment for phosphorylation of its substrate.

Could amino acids or nutrients also regulate mTORC2 activity? Depending on nutrient deprivation conditions, there are conflicting results as to whether amino acids could solely increase Akt phosphorylation.[23,66,67] Thus, the precise nutrient conditions that lead to an mTORC2-dependent Akt phosphorylation or mTORC2 activation would need to be further clarified. It would be interesting to determine if amino acids would be sufficient to promote mTORC2-ribosome association and Akt TM phosphorylation during translation. Leucine addition to starved cells promoted cell migration in an mTORC2-dependent manner, supporting the view that amino acids can activate mTORC2.[68]

The TSC1-TSC2 complex that downregulates mTORC1 is, contrastingly, required for proper activation of mTORC2.[45,69] This tumor suppressor complex physically associates with mTORC2 via rictor, but its regulation of mTORC2 appears to be independent of its GTPase-activating protein activity toward Rheb. Furthermore, the impaired mTORC2 activity in TSC2-deficient cells cannot be restored by downregulating mTORC1, suggesting that, in addition to the mTORC1-mediated feedback regulation of IRS-1, mTORC2 can be more directly regulated by TSC1-TSC2.

The GTPase Rac1 can regulate both mTORC1 and mTORC2 in response to growth factor stimulation.[70] Rac1 binds directly to mTOR independently of the GTP-bound state of Rac1 and mediates mTORC localization at specific membrane compartments. It was proposed that growth factor stimulation leads to translocation of Rac1 to the plasma membrane, recruiting mTORC2 and thereby facilitating local Rac1 activation. The Rho-GEF P-Rex1 also associates with both mTORC1 and mTORC2 but is only active when in the mTORC2 complex.[68] How P-Rex1 and Rac1 can lead to mTORC2 activation remains to be elucidated.

mTORC2 Substrates

mTORC2 phosphorylates AGC kinases at conserved motifs at the carboxyl terminal tail (C-tail) of these kinases (**Fig. 2**). The turn motif (TM) of AGC kinases that specifically contains the Thr-Pro-Pro motif, like as those found in Akt and conventional PKC (cPKC), is phosphorylated by mTORC2. It also phosphorylates the HM site that contains the Ser/Thr-Tyr/Phe sequence. The phosphorylation by mTORC2 allosterically activates the AGC kinases in addition to the catalytic activation of the kinases by PDK1. Whether non-AGC proteins can be phosphorylated by mTORC2 is also likely, since mTORC1 can phosphorylate transcription factors and the translational regulator 4E-BP in addition to the AGC kinase S6K.[11]

Akt/PKB. mTORC2 phosphorylates Akt at Thr450 of the TM and Ser473 of the HM.[60,62,63,71] Phosphorylation at the HM is induced by stimuli such as growth factors and hormones. HM phosphorylation is a post-translational modification and occurs upon recruitment of Akt to the membrane via affinity of its PH domain to membrane lipids. This would imply that mTORC2 and Akt would colocalize at the membrane. Indeed, mTORC2 has been localized to membrane compartments.[71,72] In endothelial cells, mTORC2 is recruited to lipid rafts in a syndecan-4 and PKCα-dependent manner. This recruitment to rafts is required for Akt activation in these cells.[73] HM phosphorylation allosterically activates Akt, leading to increased activity of Akt towards its many substrates including FoxO1/3. In mTORC2-deficient cells wherein HM phosphorylation is absent, FoxO1/3 phosphorylation becomes defective, whereas other Akt substrates, such as GSK3 and TSC2, remain phosphorylated.[23,24] These findings suggest that HM phosphorylation by mTORC2 may confer substrate specificity to Akt. Alternatively, other related AGC kinases could compensate in the phosphorylation of these substrates.

Phosphorylation of the HM site has been shown to be elevated in a number of cancers, indicating that the regulation of Akt by mTORC2 could play a significant role in cancer progression. In mouse genetic and xenograft studies, development of prostate cancer caused by PTEN deletion is dependent on mTORC2.[74] When several cancer cells were treated with rapamycin for prolonged periods, a subset of these cell types have reduced HM phosphorylation that correlated with disruption of mTORC2.[38] Why other cancer cells remain insensitive to this treatment is not clear, but it is also known that other protein kinases can phosphorylate the HM site.[53,75-77] Hence, abrogation of HM phosphorylation in conjunction with disruption of mTORC2 could serve as good biomarkers for tumors that can respond to mTOR inhibition.

mTORC2 also phosphorylates the TM site of Akt, but the mechanism is quite distinct from that of HM site phosphorylation. TM phosphorylation is a one-shot event that occurs exclusively during the synthesis of nascent Akt, while the polypeptide is still attached to the ribosome.[64] The association of mTORC2 with ribosomal proteins that line the rim of the ribosomal exit tunnel further supports that mTORC2 could phosphorylate this site as it emerges from the tunnel. TM phosphorylation anchors the C tail to the kinase domain and is, therefore, essential for Akt stability.[62,78] The lack of TM phosphorylation causes cotranslational ubiquitination of nascent Akt.[64] However, Hsp90 appears to rescue the stability of the majority of Akt that is not phosphorylated at the TM, since total Akt levels are only slightly attenuated in mTORC2-disrupted cells.[62,63] TM phosphorylation
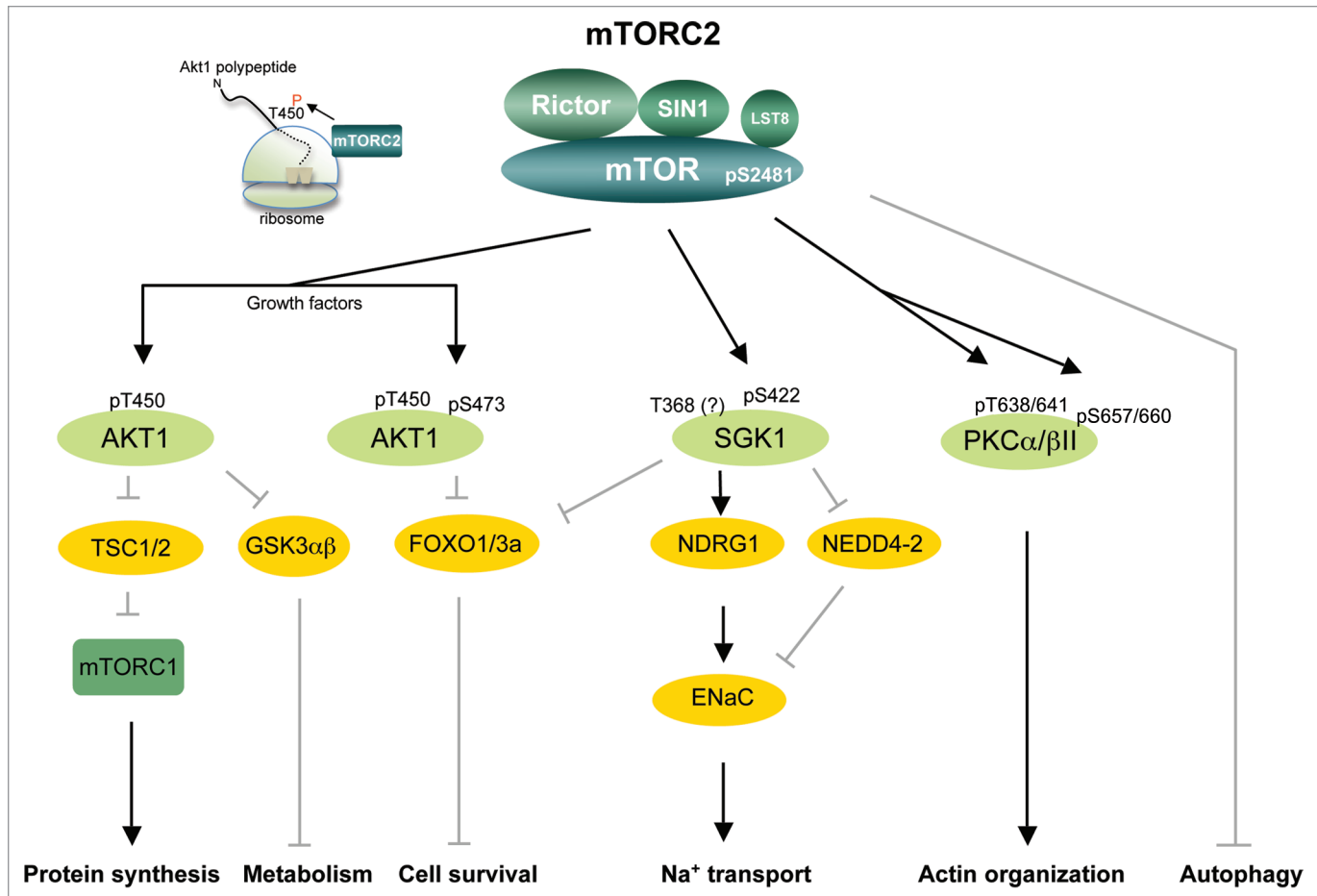
Figure 2. Functions and downstream targets of mTORC2. mTORC2 functions in a number of cellular processes by mediating the phosphorylation of conserved motifs in AGC kinases to promote their maturation, stability and allosteric activation. Phosphorylation sites that are targeted by mTORC2, mainly in the turn or hydrophobic motifs, are indicated. Catalytic activation of these AGC kinases requires phosphorylation of the activation loop by PDK1 (not shown here). In the absence of Ser473 phosphorylation, Akt can still phosphorylate some of its substrates, such as TSC2 and GSK3. AGC kinases phosphorylate a multitude of substrates that mediate numerous cellular functions. The functions of mTORC2 indicated here promote cell growth. Whether mTORC2 can play a role in cellular and organismal aging via regulation of AGC kinases or other substrates remains to be elucidated.

is quite stable, and this site does not undergo dephosphorylation once Akt has been released from the ribosomes. Whereas the HM site becomes dephosphorylated when growth signals diminish, only conditions that disrupt mTORC2 lead to absence of TM site phosphorylation in the nascent Akt polypeptide.[62-64] Since the phosphorylation of the TM is solely mediated by TORC2 from yeast to man,[62,79] this function of TORC2 has been well-conserved during evolution.

PKC. Protein Kinase C isotypes diverge in their N-terminal regulatory sequences but members of this kinase subfamily have overlapping substrate specificities.[80] They perform a variety of cellular functions but appear to play a conserved role in the spatial distribution of signals.[81] mTORC2 is implicated in the maturation and stability of PKC, primarily the conventional (cPKC) and novel (nPKC) isotypes. Phosphorylation of the TM (Thr-638/641 of PKCα/βII) and HM (Ser657/660) sites of all conventional PKC (cPKC) and some novel PKCs (nPKC) requires mTORC2.[26,62,63,82] Unlike Akt, expression levels of cPKC are pronouncedly reduced in the absence of mTORC2. In SIN1^{-/-} MEFs,

TM site phosphorylation is abolished, which could primarily account for decreased protein levels. It is also possible that mTORC2-disrupted cells have additionally decreased mRNA levels or translation of cPKC. Whether phosphorylation of the TM site is directly mediated by mTORC2 is not clear, since conventional kinase assays failed to reconstitute this phosphorylation.[63] However, when mTORC2 was added during in vitro translation of cPKC, phosphorylation of this site was detected.[64] Whether this occurs cotranslationally, as in Akt TM site phosphorylation, remains to be investigated.

HM phosphorylation at Ser657/660 of PKCα/βII is also constitutive and can occur by autophosphorylation.[83] In mTORC2-disrupted cells, HM site phosphorylation is not abolished but is attenuated.[62,63] Phosphorylation of the HM site is also important for cPKC maturation and stability and depends on interactions with Hsp90.[84] In mTORC2-disrupted cells, inhibition of Hsp90 could further diminish cPKC levels, underscoring the importance of cooperative roles of mTORC2 and folding chaperones in promoting kinase maturation and stability.

SGK. The Serum and Glucocorticoid-induced Kinase (SGK) is stimulated by growth factors and is upregulated in response to osmotic stress.[85] mTORC2 is required for HM site (Ser422) phosphorylation of SGK1.[27] Disruption of mTORC2 leads to defective SGK1 activation, which could contribute to aberrant FoxO1/3 phosphorylation. Phosphorylation of other SGK1-specific substrates such as NDRG1 was also attenuated in cells lacking rictor, SIN1, mLST8 or Protor-1.[27] Protor-1/PRR5 functions as an adaptor or enhancer of mTORC2 activity to phosphorylate the HM of SGK1.[86] The role of the TM phosphorylation in SGK1 is less clear. There are several putative phosphorylation sites around the TM. Phosphorylation at Ser397 and Ser401 located at the TM is critical for full activation of SGK1 in response to growth factors and stress stimuli.[87] A potential mTORC2-target site would be Thr368, which is part of a Thr-Pro-Pro motif and is conserved in SGK1-3. It remains to be investigated whether this is, indeed, phosphorylated and mTORC2-dependent. Unlike Akt and cPKC, SGK1 levels in mTORC2-deficient cells are not diminished. In fact, its expression is elevated in rictor-null cells.[52] Rictor, but not the other mTORC2 components, is putatively part of a functional E3 ubiquitin ligase complex that contains Cullin-1 and Rbx1. Hence, rictor could regulate SGK1 independently of mTOR. SGK1 is a short-lived protein whose N-terminal sequences are critical for Nedd4-2-mediated destruction of itself.[88] Its expression, along with its activity, may be critically monitored, given its central function in sodium transport. Indeed, mTORC2 was recently demonstrated to activate epithelial sodium channel (ENaC)-dependent Na$^+$ transport in kidney epithelial cells.[89] Furthermore, SGK1 could interact specifically with mTOR-rictor, supporting the view that mTORC2 is essential for SGK1 activation and function.

Cellular Functions

Actin cytoskeleton reorganization and cell migration. Mammalian TORC2 plays a role in actin cytoskeleton reorganization, and, similar to yeast TORC2, the Rho-type GTPases are involved in this function. Knockdown of mTOR, rictor (mAVO3), mLST8, but not raptor led to defective actin reorganization and decreased Rac1 activation upon serum restimulation.[26,90] Recently, Rac1 was found to associate with mTOR as part of both mTORC1 and mTORC2 in response to growth factor stimulation.[70] How this could mediate actin cytoskeleton reorganization remains to be elucidated, but recruitment and consequent activation of Rac1 in the plasma membrane could eventually increase PIP3 synthesis and lead to actin rearrangement. The Rho-GEF P-Rex1 also binds both mTOR complexes and promotes cell migration in an mTOR-dependent manner in response to leucine and serum restimulation. Because rapamycin does not block the leucine-induced cell migration, it was speculated that mTORC2, rather than mTORC1, participates in this process.[68] The function of mTORC2 in actin cytoskeleton reorganization can also be inferred from studies that link mTORC2 to cell migration and cancer metastasis.[91] Although the precise mechanism of how mTORC2 controls the actin cytoskeleton remains a mystery, PKC has been implicated in

this function.[26] In glioma cell lines that overexpress rictor and have increased mTORC2 activity, there was enhanced motility and elevated integrin β1 and β3 expression. The increased rictor expression correlated with elevated PKCα activity.[92] In neutrophils, chemotaxis is regulated by mTORC2 via activation of adenylyl cyclase 9 (AC9).[93] Chemoattractant-mediated activation of AC9 depends on cPKC but not Akt, hence mTORC2 could promote AC9 activity via regulation of cPKC. Rictor was found to interact with PKCζ and mediates chemotaxis and metastasis in breast cancer cells.[94] However, this rictor function appears to be mTORC2-independent and could occur when rictor expression becomes elevated. In colorectal cancer cells, both mTORC2 and mTORC1 could regulate cell motility and metastasis via the Rho GTPases. There was also elevated expression of mTORC components, including rictor, in these cancer cells.[91] mTORC2 could also control the actin cytoskeleton via more indirect mechanisms. The prostaglandin E$_2$ (PGE$_2$)-mediated mast cell chemotaxis is mediated by mTORC2.[95] mTORC2 could control this function either through actin polymerization and/or by the PGE2-induced production of the chemokine monocyte chemoattractant protein-1 (CCL2). In 3T3-L1 adipocytes, rictor that is associated with Myo1c but not mTOR participates in cortical actin remodeling.[54] This association of rictor with Myo1c is not sensitive to insulin, rapamycin or wortmannin treatment. Hence, rictor could regulate the actin cytoskeleton independently of mTOR in some cell types.

Protein synthesis and maturation. The central role of mTOR in protein synthesis is largely attributed to mTORC1[96,97] but mounting evidence points to a role for mTORC2 in this basic cellular process. Both mammalian and yeast TORC2 have been localized to membrane structure organelles such as the endoplasmic reticulum (ER) and Golgi apparatus, indicating that mTORC2 may interact with membrane-bound ribosomes.[98,99] Indeed, an intact mTORC2 localizes in polysome fractions and associates with ribosomal proteins.[64] mTORC2 components directly interact with the 60S large ribosome subunit.[64,65] Specifically, rictor can form stable associations with L23a and L26, ribosomal proteins that are positioned at the exit tunnel.[64] The nature of this interaction supports that mTORC2 could play a role in cotranslational processing or maturation of nascent polypeptides, such as Akt, that emerge from the tunnel. Identification of other nascent proteins that require mTORC2 for maturation would provide clues on mTORC2 function in the ribosomes and translation. In a yeast genetic screen, NIP7, a protein involved in ribosome biogenesis and rRNA maturation, was identified as a regulator of TORC2.[65] This led to the finding that association of mTORC2 with assembled ribosomes or ribosomal proteins activates mTORC2. Since protein synthesis inhibitors did not affect mTORC2 activity towards Akt HM phosphorylation, mTORC2 activation is perhaps independent of protein synthesis.[65] However, since enhanced PI3K activity promotes protein synthesis and increases mTORC2 association with ribosomes and mTORC2 signaling, these findings are consistent with the enhancement of protein synthesis by mTORC2. Whether translation of specific mRNAs are promoted by mTORC2 remains to be examined, but it seems that translation of mRNAs with oligopyrimidine tract at

the 5' terminus (so called TOP mRNAs that include those that encode ribosomal proteins) are not dependent on mTORC2 (nor mTORC1).[100]

Upon mTORC2 disruption or inhibition, total translation and polysomes were more severely attenuated compared to rapamycin treatment.[64,101] Studies in hematological cancers, including Philadelphia acute leukemia and multiple myeloma further confirm a role for mTORC2 in establishing or maintaining the translation machinery. When treated with OSI-027, an inhibitor of mTORC1 and mTORC2, rapamycin-insensitive leukemia cells decreased the assembly of polysomal complex.[102] OSI-027 inhibited mTOR phosphorylation at Ser2481 but not at Ser2448, suggesting involvement of mTORC2. In addition, OSI-027 impaired mRNA translation, suppressed proliferation and induced apoptosis, resulting in antileukemic responses. Notably, PP242, another dual mTOR active site inhibitor, also showed a similar effect on the active translating machinery and displayed remarkable potent anti-leukemic activity.[103,104] Thus, mTORC2 is involved in translation, and inhibiting its function simultaneously with that of mTORC1 would be more potent in inhibiting growth of cancer cells.

Consistent with the function of mTORC2 in translation, SIN1 was found to interact with an RNA binding protein, poly(rC) binding protein 2 (PCBP2), which modulates cellular responses under stress through the control of RNA stability and translation.[59] It is not known what SIN1 does to PCBP2 and whether it has a function in RNA stability as part of or independently of mTORC2.

Autophagy. Compared to rapamycin treatment, inhibition of mTOR using active site inhibitors can more potently induce autophagy. In several transformed cell lines, treatment with Torin1 or AZD8055 enhanced autophagy.[41,104,105] The effect of these mTOR inhibitors, including PP242 and OSI-027, on autophagy were also demonstrated in hematological cancers, including Philadelphia acute leukemias[102,106] and T-cell acute lymphoblastic leukemia.[104] Interestingly, in glioma xenografts and BCR-ABL-expressing leukemic cells, the pro-apoptotic effects of mTOR inhibitors were enhanced by chloroquine, an autophagy inhibitor.[102,107] This implies that cancer cells have the capacity to overcome mTOR inhibition by autophagic mechanisms that could allow cells to salvage intracellular sources of nutrients to promote growth. The extent of contribution of mTORC2 to the process of autophagy remains to be verified, since studies by Thoreen et al. found that Torin1 treatment of rictor-null MEFs led to a similar response to wild type.[41] This would suggest that enhanced autophagy may, instead, be due to inhibition of rapamycin-insensitive functions of mTORC1.

Metabolism. While the metabolic functions of mTORC1 are well characterized, little is known about the mTORC2 function in cellular metabolism.[108] A more detailed study on the role of TORC2/rictor in metabolism has been reported for *Caenorhabditis elegans*.[109] In this organism, although AKT1 and SGK1 mediate the function of TORC2 in regulating fat mass, TORC2 does not appear to be downstream of PI3K signaling for this function. In mammals, rictor-null fibroblasts were reported to display diminished overall metabolic activity.[110] In Jurkat

T leukemia cells, rictor knockdown increased mitochondrial respiration, whereas raptor knockdown decreased oxygen consumption.[111] In v-Ha-ras-transformed Pten-deficient cells that depend on mTORC2 signaling for proliferation, several genes that were required for growth were identified that mostly encode for proteins involved in mitochondrial functions.[112] The requirement for these gene products in mTORC2-addicted cells is consistent with the need for ATP production, inhibition of apoptosis and generation of products from pyrimidine synthesis, fatty acid synthesis and glutaminolysis that are all dependent on mitochondrial processes. Defining the precise role of mTORC2 in mitochondrial functions would provide important insights not only on how this pathway is crucial for altered metabolism in cancer cells, but also its role in aging.[113,114]

Tissue-Specific Functions

Whereas mTOR and raptor are absolutely required during early stages of development, mLST8 along with rictor and SIN1 are not essential during those stages.[23,24,115,116] Disruption of mTORC2 in mice by homozygous deletion of the genes for rictor, SIN1 or mLST8 also causes embryonic lethality, although embryos survive up to about E10.[23,24,110] Establishment of embryonic fibroblasts from the knockout embryos has provided compelling evidence on the cellular functions of mTORC2. Recently, tissue-specific knockout mice of the mTORC2 components have revealed novel insights on the functions of this complex in organ development and tissue homeostasis (**Fig. 3**).

Muscle. Disruption of rictor specifically in mice skeletal muscle led to impaired insulin-stimulated glucose transport. The defect in glucose transport could be due to attenuated phosphorylation of AS160, a protein that is phosphorylated by Akt and implicated in Glut4 exocytosis.[117] Another study found no alterations in muscle fiber size, and metabolic changes were found to occur upon ablation of raptor instead.[118] In both studies, the level of HM phosphorylated Akt in the muscle was reduced. However, the induction of Akt phosphorylation and activity was rescued upon combined deletion of raptor and rictor in the studies by Bentzinger et al., supporting the idea that kinases other than mTORC2 could phosphorylate Akt at the HM site. The differentiation of myoblasts was also shown to require mTORC2.[119] Rictor knockdown or prolonged rapamycin treatment that disrupted mTORC2 blocked terminal myoblast differentiation. mTORC2 performs this function via Akt, since expression of a phosphomimetic Akt can rescue myoblast differentiation. The ablation of mTOR in post-mitotic myofibers of skeletal muscles led to a more severe myopathy that led to premature death.[120] These mice displayed a similar phenotype to raptor-deficient muscles, including impaired oxidative metabolism and glycogen accumulation.

In cardiac muscle, rictor expression along with Akt phosphorylation was increased upon low-dose resveratrol treatment.[121] Resveratrol can induce an ischaemic preconditioning-like effect that is cardioprotective. This effect of resveratrol is due to the induction of autophagy, which can be inhibited by rictor knockdown. Another study that induced hypercholesterolemia in swine
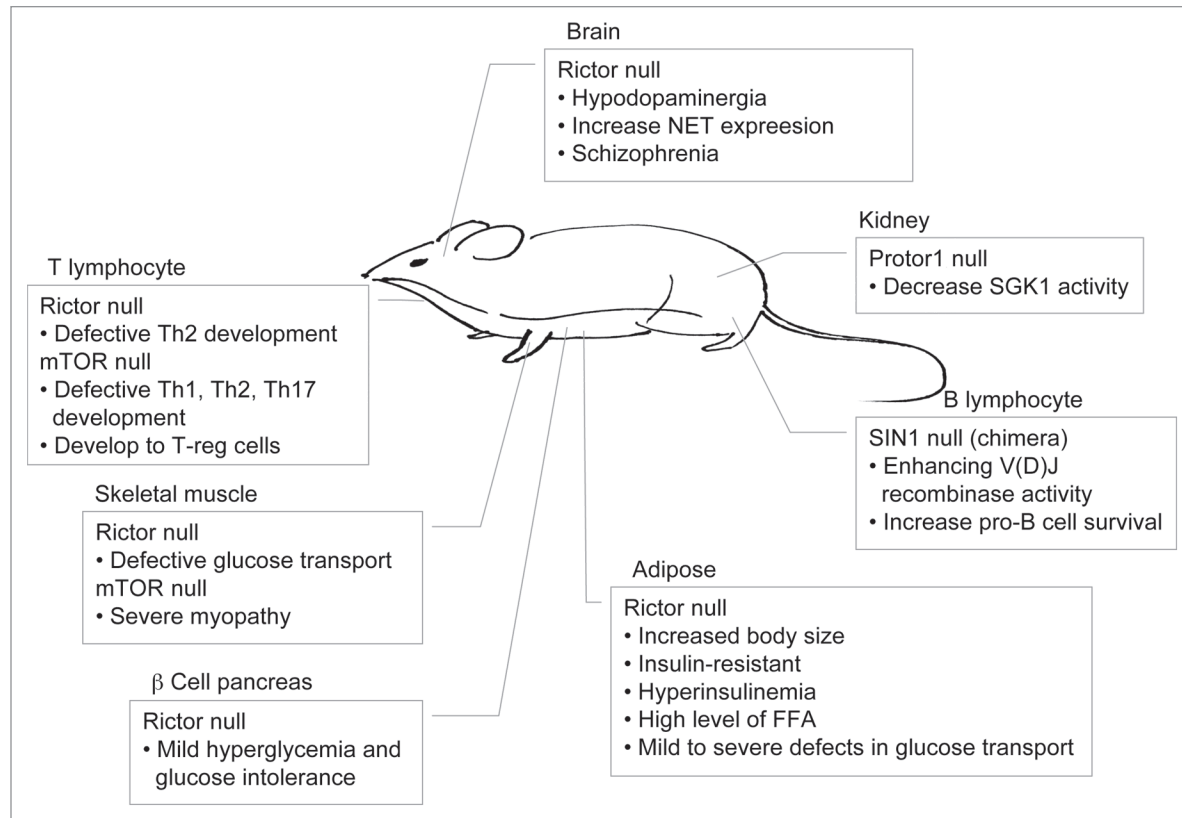
Figure 3. Tissue-specific functions of mTORC2. Genetic knockouts have revealed functions of mTORC2 in specific tissues or organs in mice.

reported increased cardiac mTORC2 activity as assessed by Akt and PKCα phosphorylation.[122] However, this was accompanied by decreased autophagy that was due to mTORC1 hyperactivation.

Adipose. A striking defect in mice with adipose-specific disruption of rictor is the increased body size.[123] This was due to increased tissue mass of organs such as the heart, kidneys, spleen, liver and pancreas, particularly on a high fat diet. These mice are modestly insulin resistant but glucose tolerant, likely due to elevated levels of insulin. The hyperinsulinemia could be attributed to increased size of pancreas and enhanced β-cell mass. These findings are consistent with a model wherein mTORC2 in the adipose regulates the production or secretion of a factor(s) that could affect growth of organs such as the liver and pancreas. The identity of this mTORC2-regulated factor remains to be investigated. In a similar adipose-specific rictor knockout, hyperinsulinemia was also reported. In contrast to the modest insulin resistance reported by Cybulski et al., a more severe systemic insulin resistance was found by Kumar et al. as assessed by hyperinsulinemic-euglycemic clamp studies. In these mice, defects in glucose uptake as well as Glut4 translocation to the plasma membrane were also observed.[124] Thus, Kumar et al. proposed that hyperinsulinemia and increased β-cell mass is mainly a consequence of the insulin resistance and elevated levels of serum-free fatty acids. In rats, chronic rapamycin treatment led to glucose intolerance and hyperlipidemia.[125] This was due to upregulation of hepatic gluconeogenesis and defective lipid deposition in adipose tissue. The decrease in adiposity can be explained by

inhibition of mTORC1-mediated PPARγ expression in adipocytes. Consistent with this interpretation, adipose-specific rictor knockout did not appear to have reduced adipogenesis or fat mass. Thus, mTORC2 functions in lipid metabolism remains to be further examined.

Pancreatic β-cell. Mice with β cell-specific deletion of rictor have mild hyperglycemia and glucose intolerance.[126] These were due to reduction in β-cell mass and proliferation causing reduced insulin synthesis and secretion. A decreased β-cell mass was also observed in rats chronically treated with rapamycin, but these animals were hyperinsulinemic due to impaired hepatic insulin clearance.[125] Interestingly, deletion of PTEN in mice had the opposite effects, leading to increased β-cell mass and proliferation.[126] Combined deletion of rictor and PTEN normalized the β-cell mass, but the size was larger due to reduced proliferation. The increased size correlated with a 12-fold increase in Akt-Thr308 phosphorylation, suggesting that, whereas the mTORC2-mediated Akt-Ser473 phosphorylation may act principally to regulate cell proliferation, Akt-Thr308 mainly regulates β-cell size (perhaps due to increased signaling to mTORC1). The enhanced distribution of FoxO1 in the nucleus in β-rictor-KO that could putatively lead to inhibition of the cell cycle could explain defective proliferation upon rictor deletion.

Brain. Both correlative and more direct genetic evidence support a role for mTORC2 in brain function. The brain has a high expression of rictor in adult mice, particularly in neurons but not in glial cells.[110] Earlier studies have linked Akt deregulation to

mental disorders, such as schizophrenia.[127] Defective dopamine signaling is thought to occur in this disorder. Impaired Akt activity terminates dopamine signaling via upregulation of the norepinephrine transporter (NET), which is responsible for dopamine clearance.[128] Neuron-specific rictor knockout mice recapitulate a key phenotype of schizophrenia, such as hypodopaminergia. An increase of NET expression in the cortex occurs in these rictor-deficient mice or upon Akt inhibition.[129] Schizophrenia-associated behavior such as prepulse inhibition deficits that could be reversed by NET blockade were observed in these rictor knockout mice, confirming a role of mTORC2/Akt pathway in the brain.

Immune system. The role of mTOR complexes in peripheral T-cell development has been described recently. Specific deletion of either mTOR or rictor at the double-positive (CD4+CD8+) and single-positive (CD4 or CD8) stages revealed that mTORC2 plays a particular role in differentiation of T helper 1 (Th1) and Th2 subsets of CD4+ cells. These two subsets develop from naïve CD4+ cells that have been activated by IFNγ/IL-12 or IL-4, respectively. Another CD4+ subset, Th17, develop upon stimulation with IL-6 and TGFβ. High concentrations of TGFβ, however, skew the development of CD4+ cells towards Foxp3+ regulatory T (T-reg) cells, which are immunosuppressive. Mice with specific deletion of mTOR in peripheral T cells displayed normal activation but failed to differentiate into Th1, Th2 or Th17 effector cells.[130] Cytokines failed to promote the upregulation of lineage-specific transcription factors, partly due to attenuated STAT activation. STAT, a family of transcription factors, was previously shown to be regulated by mTOR, although the mechanism remains obscure. Upon stimulation, mTOR-deficient T cells differentiated into Foxp3+ T-reg cells. Deletion of rictor using CD4- Cre promoter led to a more specific defect in Th2 differentiation, whereas disruption of Rheb, an mTORC1 regulator, inhibited Th1 and Th17 responses.[131] When a distal Lck-Cre promoter is used instead, a similar phenotype was observed, although fewer Th1 cells were additionally observed.[82] Thus, whereas mTORC1 is involved in Th1 and Th17 differentiation, mTORC2 appears to be specifically involved in Th2 and, to some extent, Th1 development. Precisely how mTORC2 could promote Th1 and Th2 development is not clear, but it could regulate lineage-specific transcription factors via regulation of AGC kinases. Complementation experiments in the T cell-specific rictor knockouts revealed that active Akt restored Th1 differentiation via increased expression of T-bet transcription factor. On the other hand, active PKC-θ, via GATA3 transcription factor, could rescue Th2 cell defects. The role of mTORC2 in the differentiation of other T-cell subsets including during early thymocyte development remains to be investigated.

In SIN1-/- chimeric mice, a defect in B-cell development and expansion of pro-B cells was observed. SIN1 deficiency in B lymphocytes led to an increase of IL-7 receptor and RAG recombinase gene expression, resulting in enhanced V(D)J recombinase activity and pro-B cell survival.[132] Akt2, instead of Akt1, deletion also displayed defects in *rag1* and *il7r* gene expression and attenuated FoxO1 phosphorylation. Since Akt2 is also found in the nucleus, mTORC2, via Akt2, could regulate the activity of FoxO1 in promoting the transcription of *rag1* and *il7r*. These findings imply that inhibiting mTORC2 may induce Rag expression even in mature B cells and speculatively promote genome instability and increase likelihood of generating B cell tumors. However, in vivo studies have shown that the use of active site mTOR inhibitors can effectively cause death of leukemia cells.[106] Surprisingly, although PP242 was toxic to leukemia cells, it had weaker effects on normal hematopoietic cells and mature lymphocytes. Rapamycin, on the other hand, induced immunosuppression more strongly. This may be explained by disruption of mTOR complexes upon prolonged rapamycin treatment, consistent with more severe effects on T-cell proliferation in mice with specific mTORC-disruption in T cells.[130] Active site inhibitors do not seem to disrupt complex assembly.[41] Hence, at low dosage it would be detrimental to leukemic cells but would have suboptimal effects on normal cells.

Kidney. More recently, Protor-knockout mice have been generated, and, surprisingly, these mice display no defects in Akt or PKCα phosphorylation at their HM. Instead, these mice have reduced phosphorylation in SGK1 and its physiological substrate NDRG1 specifically in the kidney.[86] These knockout studies also revealed that Protor subunits are not essential for mTORC2 complex assembly and mouse viability/growth. Since SGK1 plays a critical role in enhancing expression of epithelial sodium channels (ENaC) in the kidney, it would be interesting to determine how mTORC2, in association with Protor, can regulate salt balance under normal versus hypertensive conditions. How Protor may modulate mTORC2 activity towards SGK1 would also need to be addressed.

Conclusions and Future Direction

Studies from knockout models and mTOR active site inhibitors have revealed numerous functions for mTOR, and that some of these functions could be mediated specifically by mTORC2. Furthermore, most in vivo studies that have examined rapamycin effects actually are carried out under prolonged exposure, in which mTORC2 assembly could be disrupted. Thus, it is highly possible that some of these effects are due to mTORC2 inhibition. It would be worthwhile to re-examine the role of mTORC2 in studies that have looked at rapamycin effects on aging, immunosuppression and neurological disorders. The role that mTORC2 plays in cancer pathogenesis is highlighted by its predominant role in regulating Akt. However, it is now emerging that mTORC2 plays a more varied role that includes phosphorylation of other AGC kinases and, most likely, other cellular substrates that become deregulated in cancer.

mTORC2 is also believed to form distinct complexes that could additionally contain unique components and/or localize at specific compartments. This would provide another mechanism as to how it could perform different functions. Rictor and SIN1 can also perform functions that are independent of mTOR or of each other. Because deletion of either protein leads to instability of the other, forming a complex with another protein could be important to acquire stability and distinct function.

What are the upstream signals, in addition to growth factors and PI3K, that can regulate mTORC2, and precisely how

is mTORC2 regulated by these signals? Since yeast TORC2 is not regulated by growth factors, nutrients or stress conditions could be the more primitive regulator of TORC2. However, mTORC2 component sequences and subunits have also evolved in vertebrates and could have acquired more diversified regulatory mechanisms. Why there appears to be no close orthologs of rictor and SIN1 in photosynthetic organisms is not understood.[133]

The relationship of mTORC2 to mTORC1 and other signaling pathways is also an open line of investigation. Crosstalk between the two mTOR complexes has been found at the level of their direct substrates and other upstream regulators, thus leading to modulation of common cellular functions. Whereas several signaling pathways, including p53 and mitogen activated protein kinases (MAPK), have been reported to directly regulate mTORC1,[134-136] little is known as to how these signals can modulate mTORC2 directly. Numerous physiological functions of mTORC2 mentioned above reveal that mTORC2 could serve as an amenable target not only for malignancies, but also for the treatment of diseases such as obesity, diabetes, psychiatric illnesses and autoimmune disorders.

Acknowledgments

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